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New Possibilities of Cytostatic Drug Testing on Patient Tumor Cells by Flow Cytometry

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Summary. A new assay for cytostatic drug testing is described which can be automated. Pleural effusions and ascites are cultured as such for one week. Cells of solid tumors are cultured in the patients own serum for the same time. The cells are then stained with the esterase and intracellular pH-indicator dye 1,4-diacetoxy-2,3-dicyano-benzene (ADB) to label vital cells. They are simultaneously stained with propidium iodide (PI) as an indicator for dead cells. Monosized fluorescent latex particles are added as concentration, volume and fluorescence standard. Inflammatory cells can be distinguished in the assay from tumor cells because of their small cell volume. The number of dead and surviving cells is counted by the flow cytometer and a therapeutic index is calculated as ratio between the surviving inflammatory to surviving tumor cells. An important feature of the assay is that the DNA-distribution of the dead cells (e.g. aneuploidy) as well as the functional state of the surviving tumor cells and inflammatory cells can be judged from intracellular esterase activity and intracellular pH.

Key words: Cytostatic drug testing – Tumor cells – Flow cytometry – Esterase activity – Intracellular pH

Cytostatic drug testing on patient tumor cells is important for therapy planning but difficulties still exist with the test systems. One can broadly distinguish two types of assay. Patient tumor cells may be incubated in short term cultures with radioactive DNA precursors during the first hours after removal the body [8]. The advantage of this method is speed and closeness of the cells metabolic state to body conditions but criticism exists as to whether the radioactive label is incorporated into tumor or inflammatory cells. The practical consequence of a preferential incorporation of the label into the DNA of inflammatory cells is that the drug sensitivity of the patients inflammatory cells is determined instead of the sensitivity of his tumor cells. The short time assay is also restricted to drugs which influence cell proliferation within a few hours. This is not the case for all drugs. The tumor cell colony methods were

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developed as an alternative approach. Patient tumor cells are grown for several weeks in petri dishes [4], tubes [3] or capillaries [1] until tumor cell colonies are visible. The cultures are then incubated with cytostatic drugs. Problems arise because not all tumors form colonies in culture, and the colonies are grown under other conditions than in the body which may favor the development of new cell clones.

A new assay has been developed with the intention to maintain the culture conditions as close to the conditions in the patient as possible, and to use the possibilities of flow cytometry to measure several parameters of each cell simultaneously in order to obtain a maximum of relevant information from vital and dead cells of each assay. The parameters were, in the present study, the cell volume, the esterase activity and the intracellular pH as functional parameters of living cells and the DNA and cell volume of the dead cells.

Material and Methods

Pleural or ascites fluids of human tumors of ovarian or cervix uteri cancers were punctured under sterile conditions and cultured in 3 ml portions without additives for 7 days at 37°C in the presence of air with 5% CO_2 in petri dishes or microtiter plates. The cytostatic drugs (Doxorubicin (Adriblastin®), 4-hydroperoxy-cyclophosphamide (Endoxan®), Cisplatinum, Methotrexate, 5-Fluoruracil, Vindesinsulfate (Eldesine®), Vinblastinsulfate (Velbe®) and Actinomycin-D (Dactinomycin®) were dissolved in 10 mM TRIS/HCl pH 7.4 buffered sterile saline (TBS) and added in 60 μl quantities to give final concentrations as indicated in Fig. 2. Bone-marrow or lymph node samples were syringed several time through needles, of 1.0 and 0.75 mm diameter, washed twice in TBS, centrifuged for 10 min at 1000 × g and resuspended in the patients own ½ diluted serum at a concentration of 1×10^5 nucleated cells/ml. Solid tumors were chopped with a McIlwain tissue chopper (Mickle Lab. Engineering, Gomshall, Surrey, England) several times. The suspension was filtered with addition of TBS through a 80×80 μm nylon mesh sieve, washed and resuspended in diluted serum as bone marrow and lymph node cells. 3 ml of the final cell suspension were cultured in microtiter plates as indicated above. The cells were removed after 7 days from the culture dishes by scraping the cell layer off mechanically and by rinsing the petri dishes back and forth with TBS-buffer, dispensed rapidly with a 1 ml plastic tip pipette (Eppendorf, Hamburg, FRG) to remove remaining cells quantitatively. The cells were washed twice with TBS and resuspended in 1 ml of TBS. A 50 μl portion of each assay was cytocentrifuged and Papanicolaou stained for morphological analysis.

Of the final cell suspension 250 μl were used for flow cytometric analysis. First 5 μl of a 2 mg/ml propidium iodide solution (PI, SERVA, Heidelberg, FRG) in TBS were added to stain the DNA of dead cells. Then 5 μl of 1,4-diacetoxy-2,3-dicyano-benzene [10] (ADB, Paesel, Frankfurt/M., FRG) at a concentration of 1 mg/ml in dimethyl-formamide were added as indicator of esterase activity and intracellular pH [10] of vital cells. Staining time was 5 to 10 min at room temperature. ADB is cleaved during this time by intracellular esterases to DCH and acetate. DCH as pH indicator accumulates in the cell. The DCH and PI stain are both stable for up to 30 to 40 min at room temperature. Finally 10 μl of 5 μm diameter, NH_2 group bearing, porous and monosized particles [9], at a final concentration of 2.5×10^5 particles/ml were added as an internal concentration, fluorescence and size standard. The particles were prestained with a 20 μg/ml 1,4-dicyano-2,3-hydrochinon (DCH, Ega-Chemie, Steinheim, FRG) solution in TBS-buffer pH 7.4 for 2 h at 0°C.

The assay was measured for 2 min in a Fluvo-Metricell flow cytometer [5] with a cylindrical orifice of 85 μm diameter and 110 μm length at a current of 0.230 mA. The fluorescence of the dyes was excited between 300 to 400 nm with a HBO 100 high pressure mercury arc lamp. The blue fluorescence of DCH was collected between 418 to 440 nm and the green fluorescence of DCH and the red fluorescence of PI were collected between 500 to 680 nm. The maximum amplitude of each signal was amplified by 2.5 decade logarithmic amplifiers. The digitized amplitudes of the simultaneously measured three signals of each cell were collected ON-LINE

in list-mode [2] on magnetic tape. The tapes were evaluated in a second step with a Interdata 7/32 computer (Perkin-Elmer, Oceanport, N.J., USA, 768 kbyte core memory, 800 bpi magnetic tape, 200 Mbyte disc) with programs developed in part earlier [11]. Further details of the histogram analysis will be described in a separate communication.

Results

The three dimensional cube representation of two of the ten cytostatic drug assays (Fig. 1 a, c) of cells of an ovarian ascites (Fig. 2) show that standardization particles, inflammatory cells, tumor cells and low esterase activity dead cells can be distinguished. The cytostatic drug effect can be qualitatively seen by comparing Fig. 1 a and c where the tumor cells have practically disappeared. The projection of the cube data onto the cell volume versus esterase plane gives a good possibility for the quantitative evaluation of the data (Fig. 1 b, d). Similar histograms (not shown) were obtained for the remaining eight assays of Fig. 2. The per cent contribution of each area to the total histogram (Fig. 1 b, d) was calculated as well as the mean volumes, the intracellular pH, the mean esterase activities and the esterase to volume ratios which are a measure of the mean cellular esterase concentration. The absolute concentrations of dead and vital cells were calculated by relating the per cent contribution values of each area to the known absolute concentration of the standardization particles. All concentration values and all parameters calculated from the inflammatory and tumor cells of each assays were related to the control assays which were set as 100% (Fig. 2).

Furthermore, the ratio of inflammatory to tumor cells in each assay was calculated as therapeutic index. The therapeutic index gives an impression to what extent the cytostatic drug diminishes tumor cells and conserves inflammatory cells. Inflammatory cells are well conserved at therapeutic indices greater than 1.0 but are preferentially killed at values below 1.0. In this case of an ovarian ascites, Doxorubicin and Actinomycin-D were effective drugs because they reduced the tumor cells to 10 and 1.5% of the control values. The inflammatory cells were decreased to a lesser degree which results in high therapeutic indices of 2.5 and 8.8 (Fig. 2). Both drugs were, therefore, recommendable to the clinician. 5-Fluoruracil e.g. was not a recommendable drug. It did not diminish the tumor cells but reduced the inflammatory cells with a therapeutic index of around 0.20. The DNA distribution of the dead cells indicated that the tumor cells were euploid and no major changes of intracellular pH and esterase concentration were detected between treated and untreated cultures (not shown).

Discussion

The biological advantages of the new assay are that the culture system remains close to the conditions in the patient with regard to cellular and humoral composition of the samples. The flow cytometric method allows the distinction between small inflammatory and larger cells which are in majority tumor cells. Besides the increase and decrease of the cell numbers in the assays which are important for the therapeutic recommendation, additional information on the DNA distribution of the dead cells, the esterase activity and intracellular pH of the surviving cells and the cell volume distri-

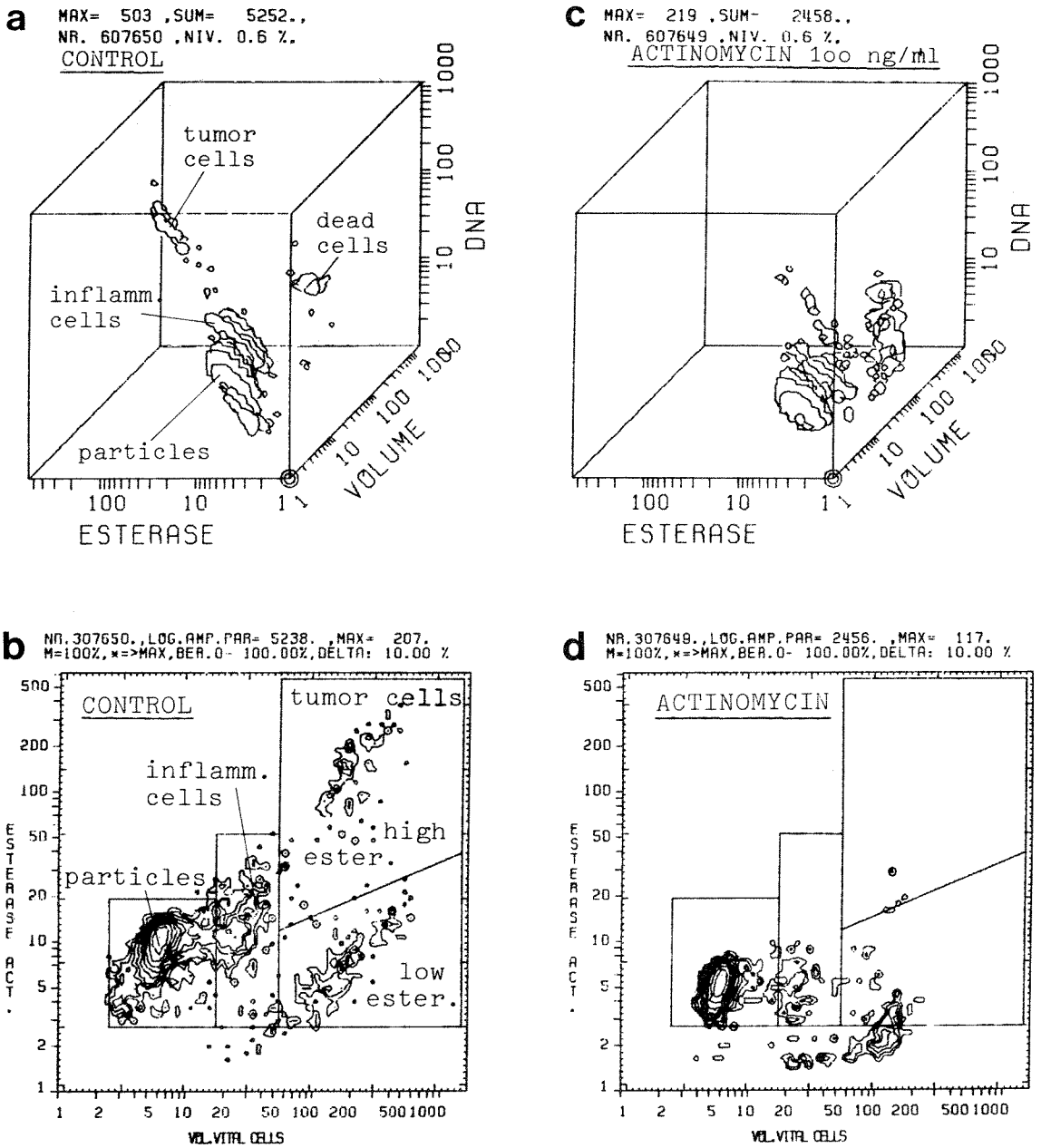

Fig. 1a–d. Three dimensional representation of a control (**a**) and an cytostatic drug assay on ovarian ascites cells with Actinomycin-D (**c**). The decrease of the frequency of the tumor cells in **c** is well visible. A total of 5262 and 2458 cells were measured respectively. The diagrams are standardized to the maximum channel contents (503, 219 particles) and the contour lines at 0.6% of the maximum channel content are plotted. The contour line is plotted around channels which contain 3 and 1 particles respectively. One obtains, therefore, the information on the position of the vast majority of all cells by this simultaneous display. The projections of the cube contents of **a, c** are displayed for quantitative evaluation in **b, d** as cell volume versus esterase activity two-parameter histograms. The logarithm of the channel content of all channels of each histogram was calculated. The maximum logarithmic channel content of each histogram was divided into 10 equal steps for each of which a contour line was plotted. The lowest contour line represents channels containing 1 cell, the highest contour line represents channel contents of 207 an 117 cells/histogram channel. The straight lines in the two parameter histograms delimit the areas for quantitative computer evaluation. The disappearence of the tumor cells in **d** is again visible. The fluorescence scales are given in relative fluorescence units. One unit of the cell volume scale corresponds to 13 μm^3. The inflammatory cells have a modal volume of 280 μm^3 and the tumor cells a volume of approximately 2600 μm^3

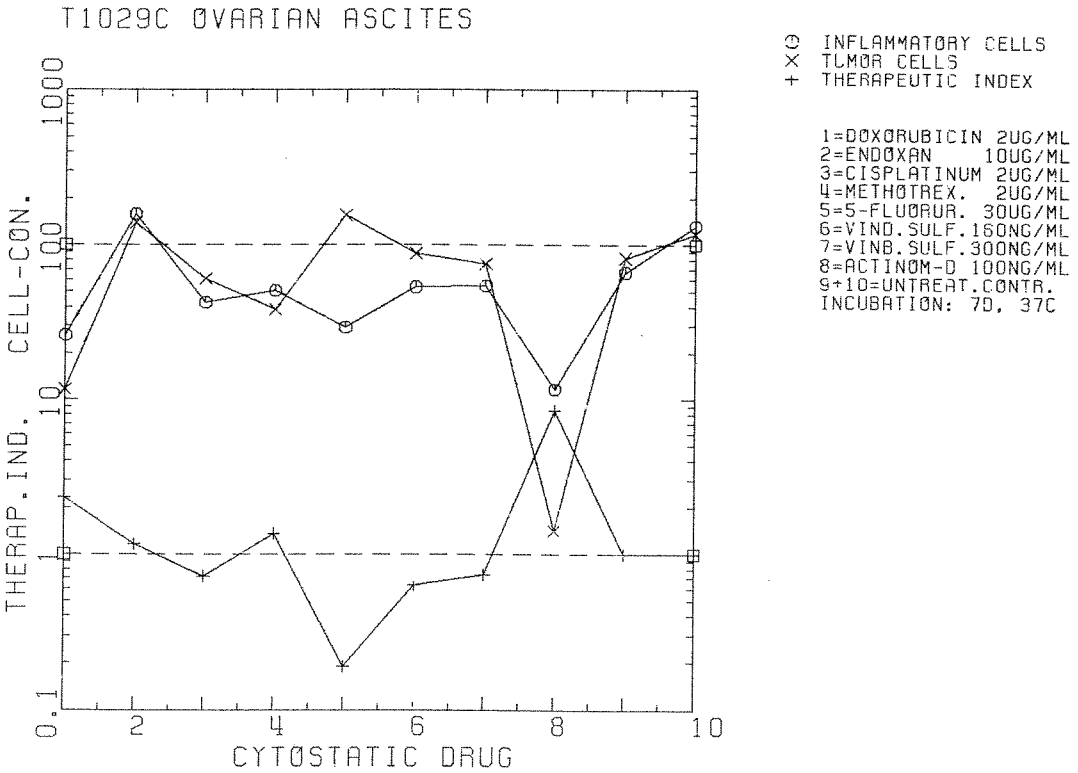

Fig. 2. Quantitative evaluation of the cytostatic drug assay of Fig. 1. The total number of surviving tumor cells (×), the inflammatory cells (○) and the therapeutic index (+) for each cytostatic drug assay (assays nr. 1–8) are expressed in per cent of the control assays (nr. 9 and 10). The therapeutic index is the ratio of the concentration inflammatory over tumor cells. Therapeutic indices (TI) > 1 indicate preferential tumor cell killing while TI < 1 are indicative of preferential killing of the inflammatory cells

bution of all cells is available. Macrophages can be identified by ingestion of rhodamin stained latex particles [7].

The tumor cells differ from the inflammatory cells mainly by their larger cell volume. One can ask if this is also a sufficient criterium to distinguish them from normal epithelial or mesothelial cells. This is directly possible when the tumor cells are aneuploid which is visible from the DNA distribution of the dead cells routinely calculated from the measurement. In case of non significant aneuploidy of the tumor cells it is not directly possible to distinguish between normal and tumor cells in the large cell compartment. The morphological examination of the cytocentrifuge preparations of all our 150 assays of ascites, pleural effusions and colorectal cancers shows, however, that overgrowth of the cultures by fibroblasts or the predominance of mesothelial cells during a 7-day period of cell culture does not occur. The majority of surviving cells under our culture conditions are tumor cells and lymphocytes. It seems, therefore, justified to use the large cell compartment as tumor cell compartment in the case of euploid tumors. The instrumental precision allows measurements with 5% to 8% coefficients of variation (CV = 100* standard deviation/mean value) of the parameters for repeated measurement of the same assay ($n = 5$, ovarian ascites). The methodological reproducibility was in the order of 10% to 15% CV ($n = 3$, ovarian ascites) when assays of the same cells were cultured in parallel. Biological reproducibi-

lity was good in a case of ovarian ascites where samples could be taken twice from the same patient within 6 weeks. Similar drug resistance patterns were obtained in both instances. An individual assay was judged to be sensitive to a cytostatic drug at a reduction of large cells to less than 50% of the control values. Preliminary evidence from all cytostatic drug tests performed, suggests that drug resistance is well predicted by the test. More than 90% of the incoming samples were assayed so far with a requirement of approximately 1 to 5×10^6 vital cells to start the cultures for 8 cytostatic drugs and the control assays. Problems arise when the tissue sample is too small or in case of uniformly coagulated ascites or pleural fluid or when heavy amorphous sediments are present in effusions. Altogether it is, however, believed that the assay is a valuable extension of the presently available methodology. It should further encourage the efforts to establish an individually planned cancer therapy.

The technical conditions of the new assay are such that staining (5 to 10 min) and measurement (1–2 min) are short and no centrifugation is required for staining. Sample staining and measurement are, therefore, suitable for automation and work in this respect is in progress in our laboratory. The calculations (Figs. 1 and 2) and plotting are presently done with the Interdata 7/32 computer but the essential calculations for the evaluation of the assay under clinical conditions can be performed in the future within a few seconds by a newly developed microprocessor data-evaluation-module [6]. The monosized particles which are included in the assay as counting reference, are important for future quality control in automated instruments since the speed of sample flow, the correct pipetting of the assay, the pH of the suspending medium and the undisturbed flow of the sample beam through the measuring chamber of the instrument can be monitored on-line by the microprocessor during the measurement. The possibility is, therefore, given to build flow-cytometers with a high degree of permanent instrument and assay monitoring, automated readjustment capabilities during the measurement and a controlled shut-down in case of orifice plugging to avoid sample loss.

It can be argued that the new possibilities of the cytostatic drug assay do not outweigh the costs of a specialized and automated instrument. This is an important point in the discussion since most of the presently available automated counters, e.g. in hematology, are in fact dedicated instruments and cannot be used for other purposes. The intention of our present technical development, in contrast, is to develop an automated multi-purpose flow-cytometer, capable of measuring cytostatic drug assays, blood samples [12] and cell samples for cancer prescreening purposes [13]. This will be possible according to our present knowledge concerning the biological and biochemical requirements of the different assays. It is also possible from the point of view data managing capacities of current microprocessors. An automated flow-cytometer of this type will be adaptable to new applications by simply changing e.g. the fluorescence filters and by loading a new program into the microprocessor memory.

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